p53 and Human Papilloma Virus Type 16 in Cervical Intraepithelial Neoplasia and Carcinoma

Bram ter Harmsel, M.D., Alex van Belkum, Ph.D., Wim Quint, Ph.D., Arie Pronk, C.T., Johan Kuippers, M.D., Ph.D., Frans Ramaekers, Ph.D., Atul Tandon, Ph.D., and Frank Smedts, M.D., Ph.D.

Summary: A series of cervical biopsies and excision specimens representing normal cervical epithelium, metaplastic epithelium, cervical intraepithelial neoplasia and cervical carcinoma were examined for the presence of p53 in relation to the human papillomavirus type 16 (HPV 16). The results show that p53 accumulation in premalignant cervical lesions is almost identical to the low levels detected in normal endo- and ectocervical epithelia, reserve cells, immature and mature squamous metaplastic epithelium. p53 levels were low and seem to be independent of the grade in cervical intraepithelial neoplasia (CIN). However, carcinomas of the cervix contained high levels of immunohistochometrically detectable p53. Apparently, a p53 mutation is not an early factor in cervical carcinogenesis. Furthermore, our studies demonstrate that alterations in p53 levels and the presence of HPV 16 are not mutually exclusive markers of cervical tumorigenesis. This contrasts with several reports in the literature and underlines the observation that p53 expression is probably an inadequate prognosticator for estimating progression or regression of CIN lesions. Key Words: p53—Immunohistochemistry—Human papillomavirus type 16—Cervical intraepithelial neoplasia—Cervical carcinoma.

The p53 tumor suppressor protein was independently discovered by three different groups in 1979 (1–3). The gene coding for this protein is located on the short arm of chromosome 17 and encodes a nuclear phosphoprotein of 53 kDa. Recent studies have shown that wild-type p53 protein has a regulatory effect on cell proliferation and transformation (4–6). p53 protein acts as a growth arrest factor, is present in low concentrations in normal cells, and has a short half-life. In contrast, mutant p53 protein has an increased half-life and therefore accumulates, primarily within the nucleus of actively proliferating cells, where it can then be detected immunohistochemically.

p53 gene mutations have a profound effect on regulatory processes governing cell division. Subtle genetic changes or more vigorous deletions enable the affected cell to grow in an uninhibited fashion, which leads to carcinomatous transformation (5). It is thought that the cell cycle is blocked at G1 with a sharp increase in the level of p53 protein. During the subsequent growth arrest, repair of DNA is completed before cells go into S phase. If the genomic damage is excessive, however, the cell undergoes programmed cell death requiring p53 protein. Cells expressing mutant p53 protein do not pause in G1 but go straight into S phase before DNA repair is complete (6). Alternatively, adequate p53 function can also be blocked by certain viral oncoproteins (7). In this respect specific human papilloma viruses (HPVs) have been implicated, and a strong correlation appears to exist between cervical cancer...
and the presence of specific HPVs. HPV types 16 and 18 are found in up to 80% of the higher grade cervical lesions and in up to 90–100% of invasive cervix carcinomas (8–10). These “high-risk” viruses encode E6 and E7 oncoproteins, which are usually expressed in cervical carcinoma and high-grade CIN. The E6 protein is capable of proteolytical inactivation of p53 (7). However, cervical carcinomas in which p53 gene mutations are detectable appear to be more aggressive than carcinomas in which p53 function is abrogated by complex formation with E6 HPV protein (8,9). Several groups have reported conflicting data with regard to the relationship between HPV and p53 mutations (10–18). Also, at present no clear relationship exists between p53 expression in a malignant cervical tumor and patient survival. Because few reports have been conducted on the presence of p53 in cervical precursor lesions and the development of p53 deficiencies during progression of CIN, an immunohistochemical study examining simultaneously the expression of p53 in HPV 16 in normal cervical tissue, dysplasias, and cervical carcinomas was undertaken.

MATERIALS AND METHODS

Tissue material

Routinely processed formalin-fixed and paraffin-embedded cervical tissue was used. The tissue samples analyzed represented diathermy loop excision specimens and cervical cone biopsy specimens from 58 women with cytologically verified dysplasia. Furthermore, 32 cases of cervical carcinoma were taken from hysterectomy specimens and excision biopsy specimens. A total of 90 cases in all were investigated. Normal endo- and ectocervical epithelia, metaplastic epithelia, the three grades of CIN, and cervical carcinoma were represented. Often more than one type of epithelium could be distinguished in a single tissue section.

p53 immunohistochemistry

The p53 antibodies used in this study were as follows:

1. Bp53-12, Biogenex, San Ramon, CA. Mouse monoclonal antibody, immunoglobulin (Ig)G2a, dilution 1:80, incubation for 1/2 h at room temperature.
2. 1801, Biogenex. Mouse monoclonal antibody, IgG1, dilution 1:60, incubation for 1.5 h at 37°C.
3. DO7, Biogenex. Mouse monoclonal antibody, IgG2b, dilution 1:40, incubation for 1.5 h at 37°C.

4. CM-1, a generous gift of Dr. D.P. Lane, Dundee, U.K. Rabbit polyclonal serum dilution 1:250, incubation for 1.5 h at 37°C.

Four-micrometer thick paraffin sections were cut, deparaffinized in xylene, and rehydrated in a descending alcohol series (95%, 70%, and 50%), after which they were desublimated in lugol for 5 min and rinsed in distilled water. Excess iodine was removed by rinsing with sodium thiosulfate and thereafter in distilled water. Endogeneous peroxidase activity was blocked by 0.3% H2O2 in distilled water for 5 min. The slides were rinsed in distilled water once more and placed in a microwave oven for two cycles of 5 min at 700 W. The sections were left to cool, rinsed in phosphate-buffered saline (PBS), pH 7.4, and incubated for 10 min with 1% bovine serum albumin (BSA) in PBS at room temperature. The p53 antibodies were as indicated above. The slides were rinsed once more in PBS for 10 min and allowed to dry, after which 1% BSA in PBS was applied. Excess BSA was removed, and immunoreactivity was detected with the Supersensitive Concentrated Detection System (Biogenex) according to the manufacturer’s instructions. After rinsing for 10 min the signal was visualized in DAB/imidazole solution. After one more rinsing step in distilled water, slides were counterstained for 5 min in hematoxylin, rinsed in tap water for 10 min, and dehydrated in an ascending alcohol series (70%, 96%, and 100%). Finally, the tissue sections were placed in xylol and mounted with a xylol-based medium (Bayer).

HPV DNA in situ hybridization

DNA in situ hybridization (DISH) was performed on all cervical tissue fragments using an adaptation of previously described protocols (19,20). Briefly, 4- to 6-μm paraffin sections were mounted on organosilane-coated glass slides. After drying at 58°C for at least 12 h, slides were deparaffinized in xylene and ethanol. Endogeneous peroxidase was inactivated by immersion for 30 min in 1% hydrogen peroxide in 50 mM Tris HCl, pH 7.6, at room temperature. After several washing steps, digestion in 0.5% proteinase K in PBS was applied for 15 h at 37°C. This critical step in DISH should be optimized for the individual tissue types. The proteinase K is then inactivated by two 3-min treatments in 0.2% glycine in PBS (pH 7). After dehydration, prehybridization was performed using a hybridization mix, consisting of 50% formamide, 2.5% herring sperm DNA, 10% dextran sulphate, 0.03 M sodium
citrate, 0.3 M sodium chloride, 0.2% BSA, 0.2% Ficoll 400 (Pharmacia), and 0.2% polyvinyl pyrrolidone (Sigma) without the HPV DNA probe for at least 30 min at 37°C. The actual hybridization step under a coverslip was preceded by a second dehy-
dration step and denaturation for 10 min at 80°C after application of the hybridization mixture. DNA probes consisted of DNase-treated cloned HPV 16 DNA, labeled with biotin using nick translation (21). The HPV 16 DNA sequences were cloned into the BamHI site of pBR322 and used as the probe during the in situ hybridization (a generous gift from Drs. Zur Ha"{u}sen and Gissmann). Probe concentration was 3 ng/µl. Hybridization was performed for 16 h at 37°C. Washing at intermediate stringency was followed by an incubation in 3% BSA in PBS. Binding of biotinylated probes was detected by coupl-
ing streptavidin conjugated to horseradish peroxidase (ICN). Detection was performed in DAB with hydrogen peroxide and imidazole. The slides were counterstained with Mayers hematoxylin, embed-
ed in Pertex (Histolab Västra, Frölunda, Sweden), and coverslipped. For evaluation of DISH specifici-
ty, all tissue sections were also subjected to hybridiz-
ation with HPV 6/11–specific DNA probes. These probes were cloned in the same way as the HPV 16 probe.

**Microscopic examination**

Stained tissue sections were microscopically ex-
aminined, and the following epithelial cell types were diagnosed: nonkeratinizing squamous epithelium (n = 41), endocervical columnar epithelium (n = 48), reserve cells (n = 22), immature (n = 21) and mature (n = 28) squamous metaplastic epithelium, as well as CIN I (n = 20), CIN II (n = 26), and CIN III (n = 23) lesions and cervical carcinomas (n = 32). Staining intensity in a lesion was semiquanti-
tively evaluated by estimating separately the rela-
tive number of positively staining cells in each type of epithelium, and furthermore the intensity of staining.

**RESULTS**

Normal cervical squamous and columnar epithe-
lium (Fig. 1a) as well as CIN lesions (Fig. 1b) gen-
erally stained weakly and only a small number of cases reacted, whereas most carcinomas (Fig. 1c) displayed strong to moderate immunoreactivity with the various p53 antibodies. Staining was mainly observed in the nuclei, although in some cases the cytoplasm in the basal cell compartment were also weakly immunoactive. The HPV DISH and the p53 immunohistochemical results were semiquantitatively analyzed, determining both inten-
sity of staining and the number of stained cells. Of the four p53 antibodies used, the strongest reac-
tivity was observed with antibody DO7, followed by Bp53-12, 1801, and CM1. HPV 16 positivity was localized in the nucleus, either diffusely throughout the whole nucleus or in a punctate fashion with one or more spots per nucleus. HPV 16 was found in the superficial epithelial cell layers, as opposed to p53 staining, which was usually located in the basal cell compartments. None of the tissue specimens re-
acted with the DNA probes aiming at HPV 6/11 DNA sequences, indicating the specificity of the HPV 16 assay and at the same time showing the absence of HPV 6/11 DNA sequences in the clinical material. For the sake of clarity, only the most salient features of the staining reactions in the various epithelia are described in Figs. 1–5.

**Normal epithelia (Figs. 1A and 2)**

In the ectocervical squamous epithelium, only scattered and weak immunoreactivity of some basal and parabasal cells was found in 14 of 17 cases with DO7 (Fig. 2A). Of these cases, only a few were stained in scattered cells with the other p53 antibo-
dies. Endocervical columnar cells were only sporad-
ically and weakly decorated in 12 of 37 cases with 
Bp53-12 (Fig. 2B). Out of this group, 10 of 36 stained with DO7, the other p53 antibodies did not decorate these cells at all. In reserve cells weak staining was observed in seven of 12 cases with Bp53-12 (Fig. 2C). The other antibodies reacted to a lesser extent in these cases. Immature squamous metaplastic epithelium displayed a variable pattern immunoreactivity in 17 of 28 cases with antibodies Bp53-12 (Fig. 2D) and DO7. Antibody 1801 stained four of 10 cases, whereas CM1 was negative in all cases. In general, mature squamous metaplastic epithe-
lium (Fig. 2E) displayed stronger immunoreac-
tivity than did immature squamous metaplasia. Staining was comparable with that of ectocervical squamous epithelium, with the exception that Bp53-
12 and 1801 stained more cases.

**CIN lesions (Figs. 1B and 3A–C)**

CIN I, II, and III displayed more or less comparable staining patterns (Fig. 3A–C) with 20 cases of CIN I, 26 cases of CIN II, and 23 cases of CIN III displaying variable but usually weak positivity in 12, 13, and 13 cases, respectively. With increasing

*Int J Gynecol Pathol. Vol. 14, No. 2, 1995*
severity of CIN there was no increase in the number of cases staining or in the intensity of immunostaining with any of the four antibodies. Those cases displaying immunoreactivity showed staining in the basal and intermediate cell layers of the epithelium, whereas the superficial layer remained negative.

Cervical carcinomas

All six keratinizing squamous cell carcinomas showed considerable levels of p53 expression when DO7 (Fig. 4A) was used. Bp53-12 staining was usually stronger, but only five of six cases were stained (Fig. 4B). The other two antibodies also stained the majority of cases. In nonkeratinizing squamous cell carcinoma, both Bp53-12 and DO7 (Fig. 4C) decorated all but one of the 13 cases examined. Staining intensity varied depending on the antibody used. Antibody 1801 and CM1 stained considerably fewer cases and as such proved to be less valuable. Approximately 75% (eight of 12) of the adenocarcinomas stained with all p53 antibodies used. Staining intensity varied from strong, e.g., in the case of a clear cell carcinoma (Fig. 4D) to weak, in a moderately differentiated adenocarcinoma (Fig. 4E). Three of the five small cell carcinomas, included in this group, showed moderate to strong p53 immunoreactivity (Fig. 4F), whereas two cases were negative (Fig. 4G). DO7 decorated all three adenosquamous carcinomas, and staining was moderate to strong (Fig. 4H). With Bp53-12, two of three cases stained moderately to strongly, whereas 1801 and CM1 only super weakly stained scattered cells in two of three and one of two cases, respectively.

Lymph node metastases of a clear cell carcinoma, an adenocarcinoma, an adenosquamous carcinoma, and a nonkeratinizing squamous cell carcinoma of the cervix were tested for p53 immunoreactivity. The primary clear cell carcinoma (Fig. 4I) was intensely positive, similar to the pattern observed in the lymph node metastasis (Fig. 4J). The
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poorly differentiated adenocarcinoma showed weak staining in both the primary tumor and metastasis. The nonkeratinizing squamous cell carcinoma showed more intense immunoreactivity in the metastasis (Fig. 4K) than in the primary tumor (Fig. 4L). The metastasis of the adenosquamous carcinoma was weakly immunoreactive, whereas the primary tumor was negative.

HPV 16 DISH

In 16 of the 90 above cases, HPV 16 positivity was observed with the DISH method (Fig. 5). Of these cases, six CIN lesions (Fig. 5A and B) and six carcinomas (Fig. 5C–F) showed immunohistochemically detectable p53. The other four CIN lesions that contained detectable amounts of HPV 16 DNA were not decorated by any of the p53 antibody preparations.

DISCUSSION

Basal levels of p53 expression and antibody sensitivity

The present study investigates the expression of p53 in an extended number of cases with normal,
FIG. 4. p53 accumulation in keratinizing squamous cell carcinoma (A and B), nonkeratinizing squamous cell carcinoma (C), clear cell carcinoma (D), adenocarcinoma (E), small cell carcinomas (F and G), adenosquamous carcinoma (H), clear cell carcinoma (I) and its lymph node metastasis (J), and nonkeratinizing squamous cell carcinoma (K) and its metastasis (L) after staining with DO7 (A, C, E, and H) Bp53-12 (B, D, F, G, K, and L), and 1801 (I and J).

Premalignant, and malignant cervical tissues. Four different p53 antibody preparations, three different monoclonals and one polyclonal serum, were applied immunohistochemically using procedures optimized for the individual reagents. Staining results were best for DO7, but reasonable levels of immunoreactivity also were observed for Bp53-12. The levels of staining of the antibodies 1801 and CM1 were considerably less, even after application of enhancement steps. Because all antibodies detect both wild and mutant types of p53 protein, it appeared that DO7 and Bp53-12 are best suited for studies on cervical tissue specimens. The two antibody preparations (1801 and CM1) used by Holm et al. (22) in a similar cervix study proved to be suboptimal in our hands. This makes comparisons with their data difficult, but may also explain that they did not detect any p53 expression in CIN and that p53 accumulation in cervical carcinoma in their study was observed in less cases than in our study.

It was surprising to note that in both normal ectocervical squamous epithelium and in normal columnar endocervical epithelium some cells contained detectable amounts of p53. This may be explained by the possibility that in normal, usually indolent cells, slightly elevated p53 levels may become detectable due to a normal physiological response to DNA damage. A less likely explanation is
that elevated levels are a consequence of p53 mutation in this morphologically apparently normal epithelium.

p53 and progression of cervical neoplasia

Reserve cells have the capacity to differentiate into immature and mature squamous epithelium, both showing low levels of p53. On the other hand, in the case of derailment of differentiation, reserve cells may transform into CIN lesions. With respect to this last route of transformation, the level of p53 staining in premalignant lesions was hardly stronger than observed in normal endo- and ectocervical epithelia. In itself this is surprising because it is well known that a certain percentage of CIN lesions are truly neoplastic, having the potential to transform into a cervical carcinoma (23). The percentage of potentially malignant CIN lesions is known to increase with the severity of dysplasia. Neither the intensity of p53 expression nor the number of cases positive for p53 increase with severity of the CIN. About 30% of cases are p53 positive, irrespective of CIN grade. At present we do not know whether the lesions demonstrating p53 accumulation are progressive or regressive in nature; this dilemma may only be solved in a prospective longitudinal study.

Most probably p53 genetic changes are late features in carcinogenesis (7). This is amply reflected by the fact that p53 expression is high in cervical carcinoma in which both the number of cells staining and the staining intensity increased in comparison with premalignant lesions or normal cervical epithelium. It is therefore felt that a change in p53 activity is not the initiating factor in cervical carcinogenesis. Expression of p53 in cervical carcinoma is high, with nearly every carcinoma (30 of 32) containing variable but easily detectable levels of p53. In this respect it would be interesting to investigate the relationship between the level of p53 accumulation and tumor grade prognosis. The important question, whether p53 staining can unequivocally and reliably indicate tumor aggressiveness, is still open.

When both primary tumors and the corresponding metastases were compared, results were conflicting. Although the number of cases is limited, primary tumors and metastases did not show concordant staining patterns. In some instances the metastases did not stain at all, whereas the original tumor was strongly decorated by all antibodies. Obviously, a larger series of cases is required to obtain insight into this matter.

p53 Expression in relation to HPV infection

In the cervix, p53 expression studies in relation to clinical parameters are few. Analysis of cell lines, obtained from cervical carcinomas, has indicated that there may be an inverse correlation between
genetic alterations in the p53 gene and the presence of HPV, particularly HPV 16 (11,13,14,17). HPV-containing cells are thought to harbor intact p53 genes, whereas cells without the tumor virus appear to be transformed due to p53 mutation. In contrast, other more clinically oriented studies document a relatively independent distribution of the p53 mutation among both HPV 16-positive and HPV 16-negative cervical tumors (12,15,18). These studies describe p53 mutations as relatively rare events (15), which may be related to the aggressiveness or metastatic potential of the affected tumor tissue (18).

HPV 16 and p53 accumulation

Of all the cases stained for p53, only 16 samples showed HPV positivity. This proportion lags considerably behind epidemiological data stating that at least 70% of cervical carcinomas should be HPV positive with DISH (20). Because we have extensively experimented with HPV staining protocols in these cases, we are not prepared to ascribe the low prevalence detected in this study to technical inadequacy. We have serious doubts regarding studies stating that a high percentage of CIN and carcinomas contain HPV 16 as detected by DISH, which in our view may be based on inadequate interpretations of positivity in general (manuscript in preparation). Presently, the DISH analysis is compared with HPV detection by polymerase chain reaction (19), but for the present the discussion will have to be limited to those cases that reacted well with the current DISH protocol. Some investigators (7,24) have reported that HPV 16 positivity in cervical dysplasia or carcinoma causes dysfunction of p53 because of complex formation with the viral E6 protein. Our study shows that p53 can be accumulated in HPV 16-positive CIN or cervical carcinoma. This finding suggests that p53 mutation and HPV 16 presence may act as independent factors in carcinogenesis.

CONCLUSIONS

The results of this study show that HPV 16 may be detected in CIN lesions and cervical carcinomas in which p53 accumulates. In premalignant cervical lesions, there is little accumulation of p53, comparable with normal ecto- and endocervical epithelia. With increasing severity of CIN, there is no evident increase in p53 accumulation, whereas high levels of p53 are observed in nearly all cases of cervical carcinoma. Of the four p53 antibodies used, two demonstrated high levels of immunostaining, whereas in two reactivity was constantly less. We conclude that p53 accumulation is not an early event in the carcinogenesis of cervical cancer, and immunodetection of the protein cannot be used as a prognosticator of malignancy in CIN lesions.

Acknowledgment: We thank Richard Schouten for his cooperation in the DISH experiments, René Bax for preparing Fig. 1, and Hanneke Drouen for helping with the layout of Figs. 2–5.

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